In vitro and *in vivo* metabolite profiling and identification of edasalonexent (CAT-1004), a bioconjugate of salicylic acid (SA) and docosahexaenoic acid (DHA) using SMART linker technology

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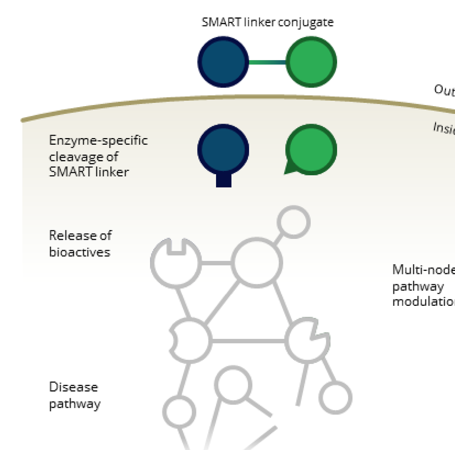
Background

Introduction

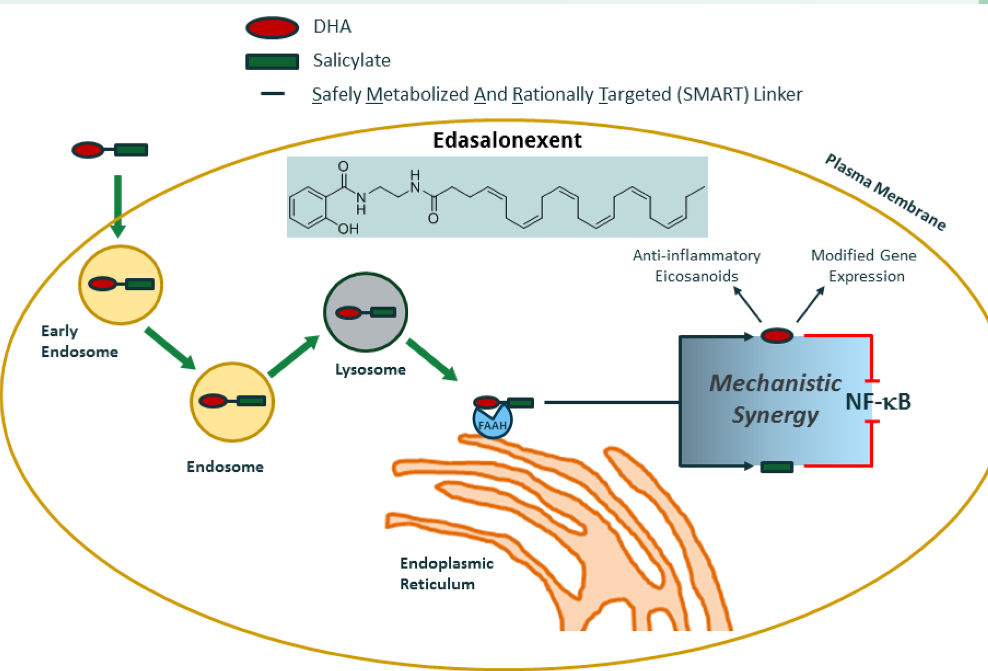
- **Duchenne Muscular Dystrophy (DMD)**
  - A progressively debilitating and ultimately fatal inherited neuromuscular disorder affecting approximately 1 in 3,500 to 5,000 live male births worldwide with a prevalence of approximately 5/100,000 in the United States
  - Caused by mutations in the gene encoding dystrophin, a critical part of the protein complex that connects the cytoskeletal actin of a muscle fiber to the extracellular matrix.
- **Edasalonexent**
  - A bioconjugate of SA and DHA using the SMART (Safely Metabolized And Rationally Targeted) linker drug discovery platform
  - Following its cellular uptake, edasalonexent was hydrolyzed into its constituents by endogenous fatty acid amide hydrolase (FAAH), simultaneously delivering SA and DHA to key intracellular targets where they inhibit NF-κB, which is activated in DMD and drives inflammation and fibrosis, muscle degeneration and suppresses muscle regeneration.
- **Clinical trials of edasalonexent in adult human subjects**
  - Three studies in adult human subjects assessed the safety, pharmacokinetics (PK), and pharmacodynamics (PD) of single or multiple edasalonexent doses up to 6000 mg
  - PK results were consistent with the intracellular cleavage of edasalonexent to its active components. Salicyluric acid (SUA), an intracellularly formed metabolite of SA (i.e., glycine conjugate of SA) was detected in plasma but SA was below lower limit quantitation. DHA was not increased in dose dependent manner. Food increased plasma exposures of edasalonexent and SUA.
- **MoveDMD trial of edasalonexent in pediatric patients**
  - 3-part, Phase 1/2, multi-site study to evaluate the safety, efficacy, PK and PD of edasalonexent in pediatric patients (≥ 4 to < 8 years of age) with a genetically confirmed diagnosis of DMD
- ***In vitro* and *in vivo* metabolite profiling and identification of edasalonexent**
  - Mouse, rat, monkey, dog, and primary human hepatocytes using CAT-1004 or ^{14}C-CAT-1004
  - Primary human skeletal myoblast cell cultures using ^{14}C-CAT-1004
  - Mouse plasma and tissues treated with CAT-1004
  - Human plasma collected from MoveDMD trial Part A

The Intersection of Pathway Biology and the SMART Linker Platform

- **Conjugates engineered from proprietary, enzyme-cleavable small chemical linkers ("SMART linkers")**
- **Cellular uptake by endocytosis**
- **Intracellular hydrolysis of linker**
- **Bioactives "reactivated" upon cleavage**
  - Released to interact with intended targets
- **Product candidates with composition of matter and method of use patents**



Edasalonexent Produces Synergistic Efficacy



Methods

Sample Collection and Processing

- ***In vitro* sample collection and processing**
  - In primary hepatocytes (1x10^6 cells/mL), CAT-1004 (10 μM) or ^{14}C-CAT-1004 with specific activity of 53.06 mCi/mmol (5 μM) was incubated for 0, 15, 30, 60 or 120 minutes
  - In primary human skeletal myoblasts (6.7x10^5 cells/mL), ^{14}C-CAT-1004 (5 μM) was incubated for 48 hours
  - Reaction was terminated with ice-cold acetonitrile containing 0.1% formic acid. Supernatant obtained from centrifugation was analyzed by liquid chromatography (LC)-mass spectrometry (MS) or LC-radiometric analysis.
- ***In vivo* nonclinical sample collection and processing**
  - Mouse plasma samples from Group 1 (control group) and Group 4 (1,000 mg of CAT-1004/kg/day) from the 7-day dose range finding study were pooled separately. Mouse plasma was protein precipitated with acetonitrile containing 0.1% formic acid, vortex mixed for 30 seconds, centrifuged at 14,000 rpm for 10 minutes at 4°C. Mouse tissue samples were also pooled by gender and group. Four equivalent mL per gram of tissues of a solution of acetonitrile/methanol (3/1 v/v) containing 0.1% of formic acid was added to the pooled tissue samples. The final mixture was homogenized using a Polytron. The homogenate was vigorously vortexed for 1 minute and then centrifuged at 4,000 rpm at 4°C for 15 minutes. Two aliquots of 1 mL of the supernatant were transferred and then centrifuged at 14,000 rpm at 4°C for 10 minutes. The resultant supernatant of plasma and tissue extraction was subject to LC-MS analysis.
- **Clinical sample collection and processing**
  - Twenty-six human plasma samples collected from Cohort A2 (67 mg/kg/day) and Cohort A3 (100 mg/kg/day) patients on Day 7 in MoveDMD trial Part A were individually (50 μL) treated with acetonitrile containing 0.1% formic acid (200 μL), vortex mixed for 30 seconds, centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant obtained was analyzed by LC-MS analysis.

HPLC Conditions

Condition 1 for samples with non-radiolabeled CAT-1004

Column	Phenomenex Gemini C6-phenyl 110Å, 2.0 x 50 mm, 3.0 μm
Guard Column	Phenomenex C_{18} 4 x 2.0 mm
Column Oven	30°C
Solvent A	H_2O + Formic acid 0.1% (v/v)
Solvent B	Acetonitrile and methanol 1:3 (v/v) containing formic acid 0.1% (v/v)
Injection volume	5 μL
Gradient	• Optimized for separation of individual identified metabolites • Flow (350 μL/min) diverted to waste from 0 to 2.0 min and from 13.0 to 16.0 min.

Condition 2 for samples incubated with ^{14}C-CAT-1004

Column	Phenomenex Kinetex Phenyl-Hexyl, 3.0 x 150 mm, 2.6 μm
Solvent A	H_2O + Formic acid 0.1% (v/v)
Solvent B	Acetonitrile and methanol 1:3 (v/v) containing formic acid 0.1% (v/v)
Gradient	• Optimized for separation of individual identified metabolites • Flow rate: 600 μL/min; split ratio 20:80, mass spectrometer: waste/fraction collector

Condition 3 for polar metabolite profiling

Column	Phenomenex HILIC 110Å, 2.1 x 150 mm, 2.6 μm
Solvent A	H_2O + Formic acid 0.1% (v/v)
Solvent B	Acetonitrile + Formic acid 0.1% (v/v)
Injection volume	10 μL
Gradient	Optimized for separation of identified polar metabolites

Radioactivity Detection, Mass Spectrometry Condition, and Metabolite Identification

Thermo Fischer Scientific Q Exactive Orbitrap

Radioactivity detection

Eluent fractions from HPLC were collected at 10-second intervals into 96-well plates containing solid scintillant. Radioactivity in each well was determined using TopCount analysis, and radiochromatographic profiles were generated based on radioactivity counts.

Mass spectrometry condition:

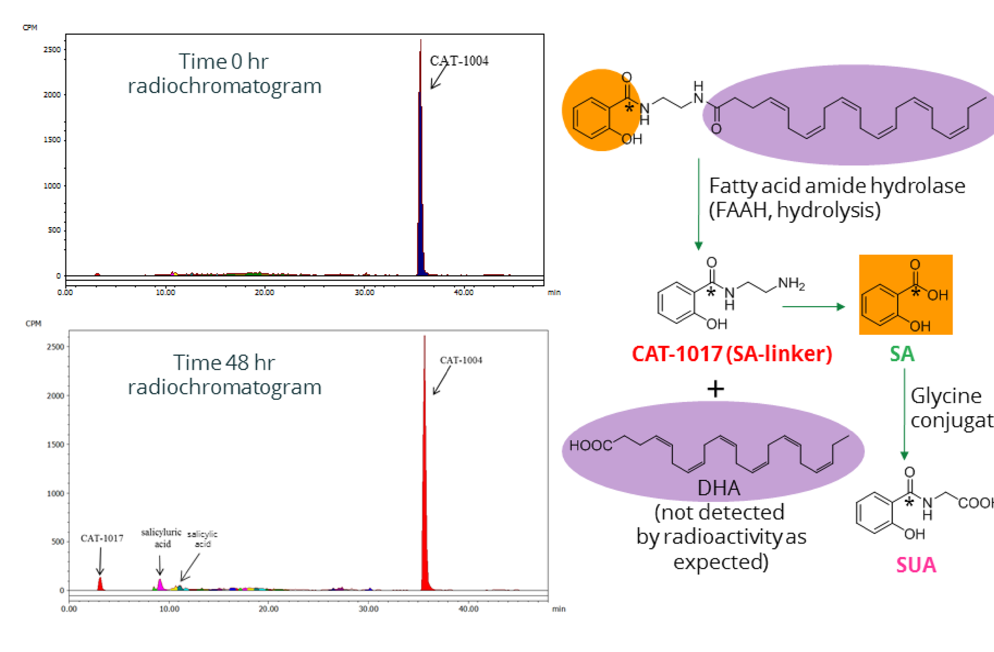
Ionization Interface	Positive/negative heated electrospray
Survey Scan	m/z 100 – 1500 at 70,000 or 100 - 1000 at 140,000 resolution
Dependent Scan	MS^2 17,500 or 35,000 resolution
Source Voltage	4.0 kV positive, -2.4 kV negative
Collision Energy	35
S-lens RF Level	40 or 80
Capillary Temperature	325 or 360 °C
Source Temperature	425 °C

Metabolite identification based on:

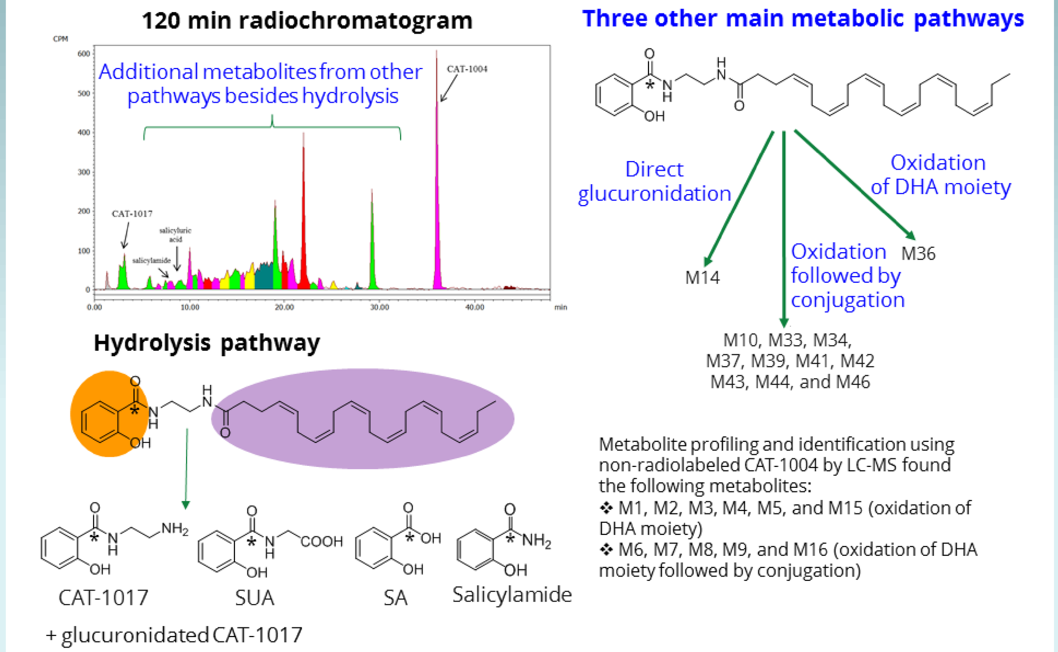
- ✓ The accurate mass determination of the pseudo-molecular ion,
- ✓ Absence of the pseudo-molecular ion at the same retention time in blank control sample(s),
- ✓ Agreement between the theoretical and the measured isotope pattern based on the proposed metabolite chemical formula,
- ✓ MS/MS spectrum consistent with the predicted metabolite structure, and
- ✓ Retention time of the available reference standards

Results

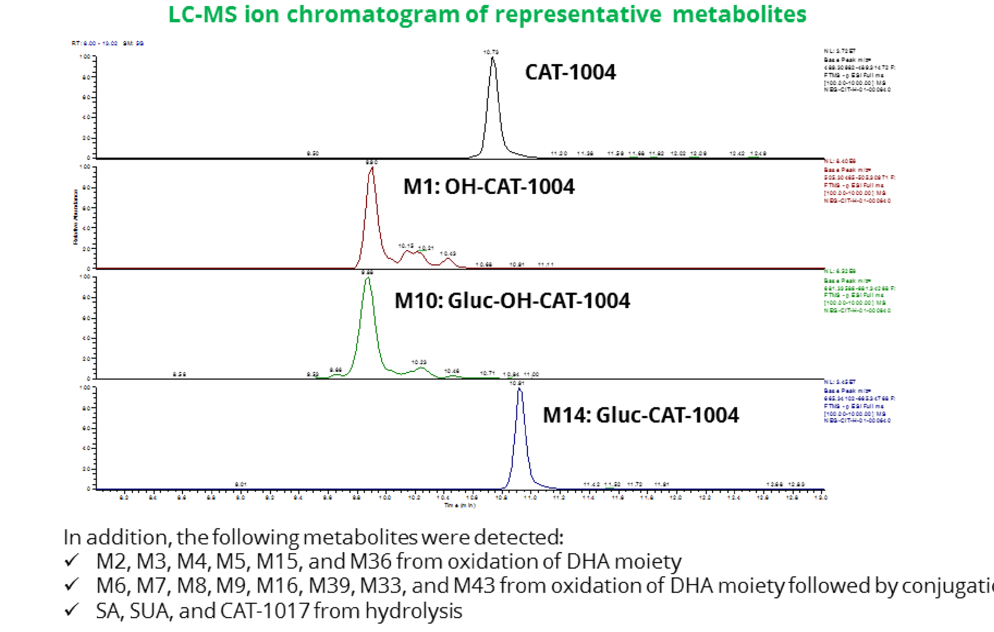
Metabolite Profiling and Identification in Primary Human Skeletal Myoblasts



Metabolite Profiling and Identification in Primary Human Hepatocytes



Metabolite Profiling and Identification in Human Plasma



In addition, the following metabolites were detected:

- ✓ M2, M3, M4, M5, M15, and M36 from oxidation of DHA moiety
- ✓ M6, M7, M8, M9, M16, M39, M33, and M43 from oxidation of DHA moiety followed by conjugation
- ✓ SA, SUA, and CAT-1017 from hydrolysis

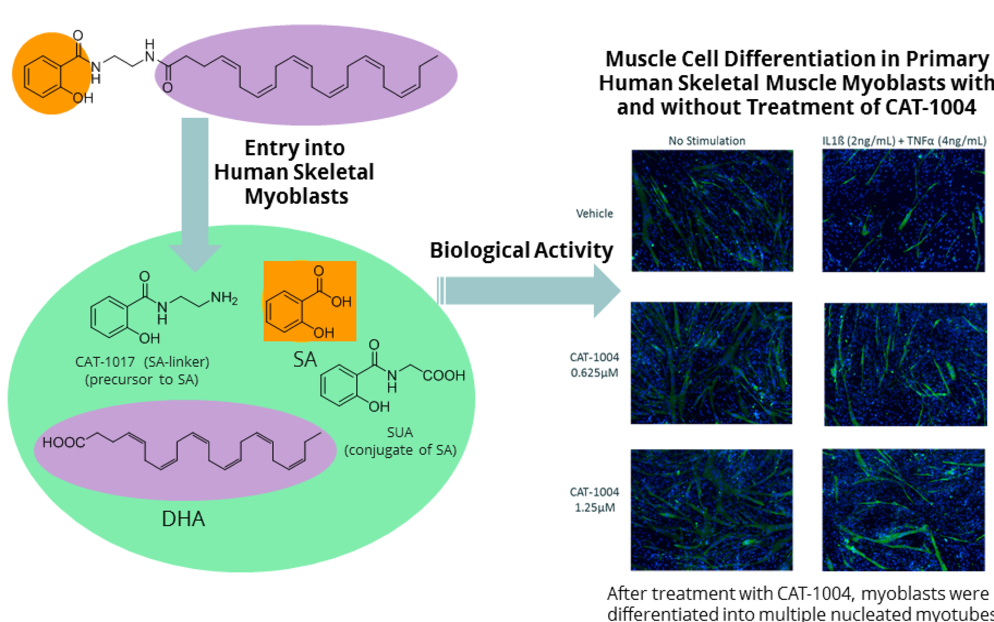
Cross-Species *In Vitro* and *In Vivo* Edasalonexent Metabolism Comparison

Metabolites detected in human plasma	Detected in nonclinical species *in vitro* or *in vivo*
M1	RH DH MkH MoP MoAt MoLt MoMt
M2	DH MkH MoP MoLt
M3	RH DH MkH
M4	MkH MoP MoAt MoHt MoKt MoLt MoMt
M5	MoP MoKt MoLt
M6	DH MkH MoP MoLt
M7	DH MkH MoP MoKt MoLt
M8	MkH MoP MoAt MoKt MoLt MoMt
M9	MoP MoAt MoKt MoLt
M10	DH MkH MoP MoAt MoHt MoKt MoLt
M14	RH DH MkH MoP MoAt MoHt MoKt MoLt MoMt
M15	DH MkH MoP
M16	DH MkH MoP MoLt
M18 (salicyluric acid)	RH DH MkH MoP MoHt MoKt MoLt MoMt
M26 (CAT-1017)	MoH, RH DH MkH MoP MoAt MoHt MoMt
M33	MoH, RH MoP
M35 (salicylic acid)	DH
M36	MoH, RH DH
M39	DH
M43	DH

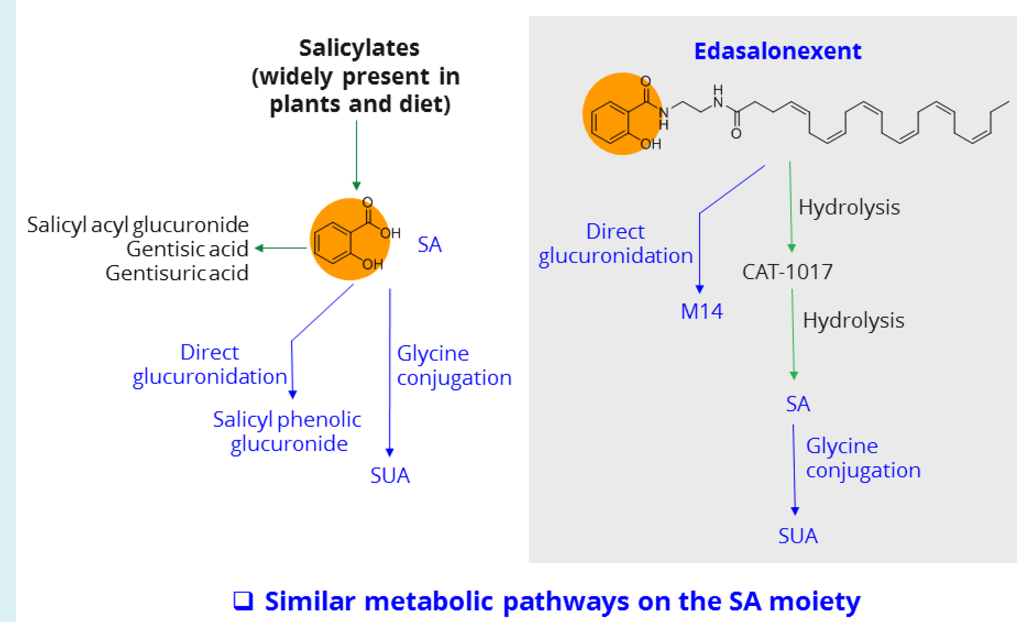
MoH: mouse hepatocytes
RH: rat hepatocytes
DH: dog hepatocytes
MkH: monkey hepatocytes
MoP: mouse plasma
MoHt: mouse heart tissue
MoAt: mouse adrenal tissue
MoMt: mouse muscle tissue
MoKt: mouse kidney tissue
MoLt: mouse liver tissue

Discussion

Demonstration of SMART Linker Technology in the Target Human Cells and Intersection with Biological Activity

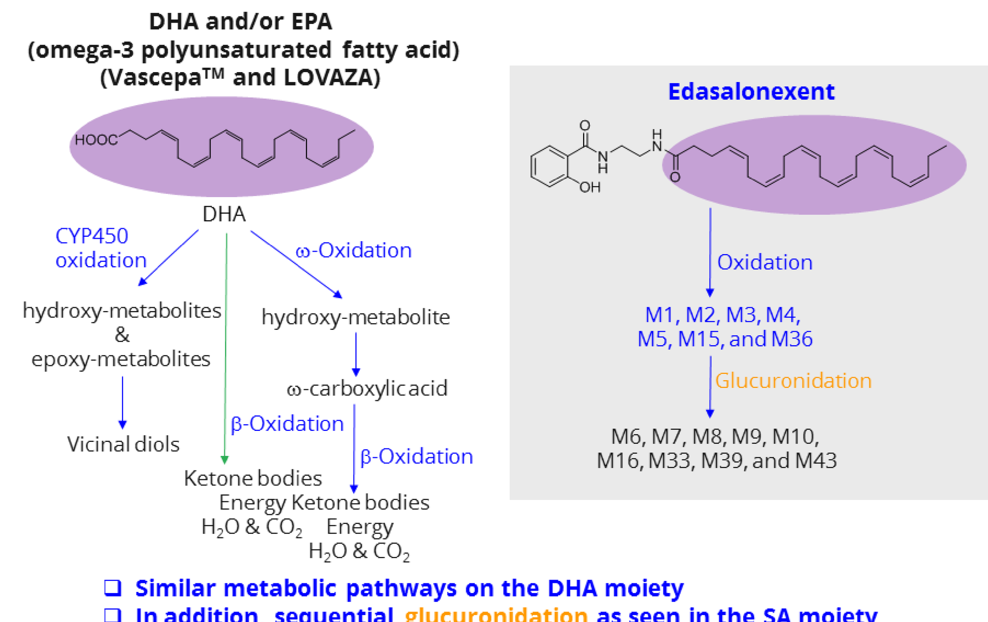


Metabolic Pathway Comparison of Edasalonexent with Salicylates



❑ **Similar metabolic pathways on the SA moiety**

Metabolic Pathway Comparison of Edasalonexent with Drugs Containing DHA and/or EPA



❑ **Similar metabolic pathways on the DHA moiety**
❑ **In addition, sequential glucuronidation as seen in the SA moiety**

Conclusion

- **In primary human skeletal myoblasts, edasalonexent was metabolized intracellularly and released the two known bioactives and the sequential metabolite of SA via glycine conjugation, demonstrating experimentally the core principle of the SMART linker technology platform.**
- **In primary human hepatocytes, besides releasing the two bioactives via hydrolysis, edasalonexent also underwent oxidative metabolism of DHA moiety, oxidative metabolism of DHA moiety followed by conjugation, and direct glucuronidation.**
- ***In vivo* metabolic profile and characterization of edasalonexent in human plasma and mouse plasma/tissues were in general consistent with the *in vitro* metabolic profiles.**
- **Cross-species *in vitro* and *in vivo* drug metabolism comparisons revealed all metabolites identified in human plasma using LC-MS were present in at least one nonclinical safety evaluation species.**
- **The metabolism of DHA moiety in edasalonexent was consistent with the metabolic pathways of omega-3 polyunsaturated fatty acids reported in the literature. The direct glucuronidation of edasalonexent was also consistent with the metabolic pathway of the salicylates observed in human.**

Acknowledgments

– **Patients and Families**
– **Patient groups**
– **Catabasis team**

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